(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 March 2002 (14.03.2002)

PCT

(10) International Publication Number
WO 02/20571 A2

(51) International Patent Classification[7]: C07K 14/16

(21) International Application Number: PCT/EP01/10244

(22) International Filing Date:
5 September 2001 (05.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00203116.9 8 September 2000 (08.09.2000) EP

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(81) Designated States *(national)*: AE, AG, AL, AU, BA, BB, BG, BR, BZ, CA, CN, CO, CR, CU, CZ, DM, DZ, EC, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MA, MG, MK, MN, MX, MZ, NO, NZ, PH, PL, RO, RU, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ZA.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/20571 A2

(54) Title: ATTENUATED HIV STRAINS AND USE THEREOF

(57) Abstract: The invention provides an isolated human immunodeficiency virus, comprising at least one non revertant mutation capable of delaying or diminishing the pathological behavior of said immunodeficiency virus when compared to a human immunodeficiency virus not having at least one such a mutation. A virus of the invention can be used for the preparation of a vaccine for Aids. Said virus can also be used for diagnostic assays in HIV-infected patients.

Title: Attenuated HIV strains and use thereof

The present invention relates to the field of immunology, in particular to viruses and more in particular to human immunodeficiency virus.
Live-attenuated virus vaccines have been enormously successful. They are widely used to prevent diseases like for instance polio and measles. Until now, however, there is no vaccine against acquired immunodeficiency syndrome (Aids). All over the world much research is being done with human immunodeficiency virus to obtain a suitable vaccine. Although attenuated strains have been obtained, there still remain many safety concerns about either the reversion of attenuated vaccine strains to virulent phenotypes or the induction of fulminant infection in (immunocompromised) individuals. An example of the possibility of attenuated strains to regain their pathological behavior is a recent publication of Berkhout et al. They demonstrated that the HIV-1 delta3 vaccine candidate, which contains 3 deletions in non-essential parts of the genome, is able to regain full replication capacity within four months of replication in tissue culture (Berkhout et al., 1999). Another proof of the genetic instability of attenuated strains is the finding by Baba et al that deletion variants of the simian immunodeficiency virus (SIV) showed an increased ability to replicate after several years in some infected monkeys, concomitant with the onset of Aids (Baba et al., 1999). Furthermore, some individuals with a vaccine comprising attenuated HIV-1 variants lacking the *nef* gene recently showed a decline in CD4+ T-cell numbers, indicating that these individuals could develop Aids (Dyer et al., 1999; Greenough et al., 1999). So until now there is no suitable vaccine with live-attenuated HIV. This kind of vaccine is to be preferred, however, because other vaccines comprising inactivated viruses or subunits do not result in a broad-based immune response or long-term memory necessary to confer life-long protection in immunized individuals. Therefore life-attenuated HIV vaccines are still under investigation.

The present invention discloses the unexpected and important finding that certain non revertant mutations in a human immunodeficiency virus are capable of delaying or diminishing the pathological behavior of said virus for a very long time *in vivo*. We have isolated and sequenced said mutant human immunodeficiency viruses, derived from a patient which lacks the characteristic decline in CD4+ T cell number, informed consent. The individual that carried the HIV virus with the mutations described in tables 1 through 4 was relatively healthy with high CD4+ cell counts in the blood. This phenomenon is uncommon in HIV infection where normally a significant drop in CD4+ cell count is observed. In this respect seemed the HIV virus that infected the patient less or even non-pathogenic. The HIV virus was, however, immunogenic as shown by the seroconversion of the individual. Furthermore, experiments with strains of said virus *in vitro* showed a normal growing pattern compared to human immunodeficiency viruses not having at least one such a mutation (figure 2). These are suitable characteristics for a virus suited for vaccine development as life attenuated vaccine. Figure 1 shows the detected amount of HIV-RNA and CD4+ T cells in said patient during the last five years.

Thus, the present invention discloses an isolated human immunodeficiency virus, comprising at least one non revertant mutation capable of delaying or diminishing the pathological behavior of said immunodeficiency virus when compared to a human immunodeficiency virus not having at least one such a mutation. Preferably, a virus of the invention is a HIV-1 virus. A non revertant mutation is defined as a mutation which is stable and remains present in the virus over a prolonged period of time. Preferably, said non revertant mutation is stable and remains present in the virus over a prolonged period of time in a patient. By delaying the pathological behavior of said virus is meant that it takes a longer time after primary infection before the amount of CD4+ T cells in the infected individual starts to

decline compared to the time it takes with a human immunodeficiency virus not having at least one such a mutation. Diminishing the pathological behavior of said virus is defined as decreasing a capability of said virus to significantly reduce the number of CD4+ T cells in an individual infected with said virus. Significantly reducing is defined as reducing said number of CD4+ T cells more than during a normal variation within said individual. Preferably, a virus of the invention comprises at least one amino acid sequence as is described in tables 1 and 2. Thus in one aspect the invention provides a virus of the invention, comprising at least one amino acid sequence as described in table 1 or 2. In another aspect the invention provides a virus of the invention, comprising at least one amino acid sequence as described in table 1. In a preferred embodiment the invention discloses an isolated virus according to the invention, wherein at least one of said non revertant mutations is located in the *gag* or *pol* gene. Important mutations are the 3 amino acid (QAE) and 10 amino acid (QSRPEPTAPP) insertions and the 2 amino acid deletion in the *gag* gene and the "IPIK" mutation in the *pol* gene.

Alternatively, a virus of the invention may comprise at least one substitution amino acid in an amino acid sequence as described in table 1 or 2. Said substitution amino acid is defined as an amino acid which does not substantially alter the capability of said amino acid sequence of delaying or diminishing the pathological behavior of a virus of the invention when compared to a human immunodeficiency virus not having at least one such a mutation. Thus, in another aspect the invention provides a virus of the invention, which comprises at least one substitution amino acid in at least one amino acid sequence as described in table 1 or 2.

A virus of the invention is obtainable by state of the art cloning techniques. A person skilled in the art knows a variety of ways to perform site directed mutagenesis. Thus, the present invention also discloses a method for

obtaining a virus according to the invention, comprising providing a wild type human immunodeficiency virus with at least one non revertant mutation capable of delaying or diminishing the pathological behavior of said immunodeficiency virus when compared to a human immunodeficiecy virus not having at least one such a mutation.

Alternatively, a virus strain of the invention can be isolated by randomly collecting human immunodeficiency strains and selecting for strains comprising sequence similarities to a virus according to the invention. By sequence similarity is meant that the isolated strains comprise at least one same mutation as a virus according to the invention, said mutation being capable of delaying or diminishing the pathological behavior of said isolated virus when compared to a human immunodeficiency virus not having at least one such a mutation. Said isolated virus may contain additional mutations. Said additional mutation may also be involved in the delaying or diminishing of the pathological behavior of said isolated virus when compared to a human immunodeficiency virus not having at least one such a mutation. Said additional mutation may render said isolated virus even more attenuated. Thus, in another embodiment the invention provides a method for obtaining a virus of the invention comprising collecting a certain number of strains, sequencing at least part of said strains, comparing obtained sequences with sequences of virus according to the invention, and isolating strains comprising sequence similarities to a virus according to the invention. In another embodiment, said strain is amplified before sequencing in said method.

Of course, a method of the invention is particularly useful for obtaining an attenuated virus according to the invention. Therefore, in another aspect the invention provides a virus obtainable by a method according to the invention.

A virus of the invention may be used to prepare a vaccine. If administered to an immunocompetent individual, said individual will develop antibodies against HIV. Said antibodies give the individual at least a partial

protection against more virulent strains. Thus, the invention provides a virus according to the invention for use as a vaccine. As the strains that we isolated thus far are still capable of reducing the number of CD4+ T cells in an individual infected with said strains, a virus of the invention is preferably processed further. In combination with other changes in the HIV genome the mutations described in tables 1 through 4, or a selection thereof, can be used for the design of a safe live attenuated HIV vaccine. In addition, the same mutations can be used in vaccines composed of dead virus, virus without replicatable nucleic acid or protein subunits. These mutations have shown to be immunogenic and provoke an immune response capable of suppressing the growth of the HIV virus. For part this will be the result of features of the individuals immune system, but another, equally essential, part is the HIV virus part. The immunogenic determinants of the proteins play a central role in the quality and characteristics of the evoked immune response.

Thus in another aspect the present invention provides a use of a virus according to the invention for the preparation of a vaccine. Of course, said vaccine will specifically at least partly provide an individual with protection against Aids. Thus, the invention discloses a use of a virus according to the invention for the preparation of a vaccine for Aids. In yet another aspect, the invention discloses a vaccine comprising virus according to the invention. A vaccine of the invention is particularly useful for prophylaxis of Aids. Therefore, the present invention provides a method for, at least in part, prophylaxis of Aids, comprising administering a vaccine according to the invention to an individual.

With the teaching of the present invention, a person skilled in the art is capable of identifying a virus of the invention in an individual. Mutations comprised by a virus of the invention can be used as target sequences for diagnostic assays to discriminate HIV sequences with and without the

mutations from tables 1 through 4. Diagnostics capable of identifying these mutations may play a role in assessing the life expectancy of infected individuals, whereas these mutations or a subset thereof indicates a better quality of life and a longer disease free period compared to other HIV viruses. Therefore, another embodiment of the invention discloses a method for identifying a virus of the invention in an individual, comprising collecting a sample comprising virus or parts thereof, from said individual.and detecting strains comprising sequence similarities to a virus of the invention. Preferably, said sample is a plasma, serum or blood sample. Virus may be collected from an individual by collecting blood samples comprising peripheral blood monocytic cells (PBMC). Thus, another embodiment discloses a method of the invention, wherein said virus is collected by isolating peripheral blood monocytic cells from said individual.

Sequence similarities are defined as before in this description. A person skilled in the art is able to determine sequence similarities. For instance, he/she is able to detect a virus of the invention using antibodies with a binding specificity for one or more of the stable mutations of said virus. Alternatively, a person skilled in the art can detect sequence similarities by sequencing collected virus from an individual. Techniques of sequencing are well known in the art. Thus, another embodiment of the invention discloses a method according to the invention, wherein said sequence similarities are detected by sequencing.

Of course, there are other possibilities to detect sequence similarities between an isolated strain and a virus of the invention. One possibility is for example hybridization with probes comprising at least one sequence of virus according to the invention. Thus, yet another embodiment of the invention provides a method according to the invention, wherein said sequence similarities are detected by hybridization with probes comprising at least one sequence of virus according to the invention. A person skilled in the art can think of other possibilities to detect sequence similarities between an isolated

strain and a virus of the invention. If another way of detecting is used in a method of the invention, it is still within the scope of the present invention.

The following, non-limiting, examples illustrate the present invention. They are not limiting the invention in any way. With the teaching of the present invention, a person skilled in the art can perform alternative experiments which are still in the scope of the present invention.

Example 1.

In this example we describe the sequencing of full genome sequences of HIV-1.

The method is build up of the following steps.

1. Preparation of GAT (generic amplification tool) mixtures:

Mix A

Solution	1x (µl)
10x PCR buffer II	2
100mM $MgCl_2$	1
100µM dNTPÁs	0,8
100ng/µl oligo JZH2R	0,5
H_2O	4,7
RNAsin **	0,5
50U/µl MuLV-RT *	0,5

*Add the enzyme before use.

** For DNA sequencing no RNAsin in mixture

- 10x PCR buffer II (500mM KCl, 100mM Tris-HCl, pH8.3; included in kit Perkin Elmer; catno: N808-0161).
- RNAsin (Perkin Elmer N808-0119).
- MuLV-RT (Perkin Elmer N808-0018).
- JZH2R primer: 5Á- GCT ATC ATC ACA ATG GAC NNN NNG , 3Á

Mix B

Solution	1x (µl)
10x seq. buffer2	4
100µM dNTPÁs	1,6
100ng/µl oligo JZH2R	1
H_2O	13,2
13U/µl Sequenase 2.0 *	0,2

*Add the enzyme just before use.

- 10x seq. buffer2 (350mM Tris-HCl pH7.5, 175mM $MgCl_2$, 250mM NaCl).
- Sequenase 2.0 (Amersham, USB 70775)

PCR mix

Solution	1x (µl)
10X PCR buffer	5,0
100mM $MgCl_2$	0,9
100µM dNTPÁs	0,2
100ng/µl JZH1	1,0
H_2O	40,6
5U/µl Amplitaq	0,3

- Amplitaq (Perkin Elmer N808-0161).
- JZH1 primer: 5Á- GCT ATC ATC ACA ATG GAC , 3Á

2. Isolation of Nucleic Acid

- 10 µl of **culture supernatant** is used to isolate the nucleic acid with Protocol Y described by Boom et al., J Clin Microbiol. 1990 Mar;28(3):495-503.
- elute the nucleic acid with 30µl H_2O.

3. GAT

*1. First strand synthesis**

- Take 10µl of PROTOCOL Y/Sc[1] isolated product.
- Incubate 5 minutes at 80°C , quench on ice.
- Add 10 µl mix A (JZH2R; MuLV-RT, add enzyme before use).
- Incubate 10 minutes at room temperature.
- Incubate 30 minutes at 42°C.
- Incubate 5 minutes at 80°C, subsequently cool down to room temperature.
- Add 0.5µl RNAse-H (1U/µl; Boehringer Mannheim,; 786357).
- Incubate 30 minutes at 37°C.

2. Second strand synthesis

- Take 20µl of the first strand synthesis (keep it on ice).
- Add 20µl of mix B (JZH2R; Sequenase 2.0, add enzyme before use).
- Incubate 10 minutes on ice.
- Incubate 10 minutes at room temperature.
- Incubate 30 minutes at 37°C.
- Store either on ice for following amplification or in the ,80 for later use. Best is to perform the PCR immediately after the first and second synthesis.
- 2µl of product is used for PCR.

*3. PCR**

- 48µl PCR-mix (JZH1).
- Add 2µl of GAT product..

4. PCR Program (Perkin Elmer 9700 PCR machine).

- 5Á 95°C.
- 20ÁÁ 95°C, 30ÁÁ 55°C, 2Á 72°C for 45 cycles.
- 10Á 72°C.
- 10Á 4°C.

15 µl is examined on 1.2% agarose gel and the method was considered te be successful if long smears could be observed in the gel.

Dilute GAT product for multiple specific HIV1 PCR reactions. Standard dilution rate as input for the amplification is 10 times (10 øl GAT product + 90 øl Baker water) or 100 times (10 øl GAT product + 990 øl Baker water). Usually a dilution rate of 100 times generates the best results. Therefore first the 100 times dilution is used for amplification. If the result is not satisfactory an additional amplification on the 10 times dilution is done.

Subsequently perform 20 specific HIV1 PCR reactions (see list for primer sets and details) according to standard PCR amplification specifications.

5' PRIMER	3' PRIMER	LENGTH	SEQPRIMER
5' PROT I	3' PROTII	572	PROT FM
5'V3NOT	3' ENV KN	1628	L2
5'V3NOT	3' ENV KN	1628	3' ENV KN
5'V3NOT	3' ENV KN	1628	L3
5'V3NOT	3' ENV KN	1600	WS9rev
5'V3NOT	3' ENV KN	1600	GP120 3'PCR
5'V3NOT	L9	900	L9
5'V3NOT	L9	900	5'V3NOT
FGS001	FGS002	670	FGS002
FGS001	FGS002	670	FGS001
FGS003	FGS004	688	FGS004
FGS003	FGS004	688	FGS003
FGS005	FGS006	535	FGS006
FGS005	FGS006	535	FGS005
FGS005	FGS008	1095	FGS007
LOUW-1-GAG	SK39	900	LOUW-1-GAG
LOUW-1-GAG	SK39	900	SK431
LOUW-1-GAG	SK39	900	GAGAE-3T7
POL 5'FM	VPR3-T7	1050	POL5'FM
POL 5'FM	VPR3-T7	1050	VPR3-T7
PROT FM	3'HALFRT	1500	SP6 P66
PROT FM	3'HALFRT	1500	ENDPROTT7
PROT FM	3'HALFRT	1500	3'HALFRT
PROT FM	ENDPROTT7	1000	PROT FM
RT19new	3' HALFRT	1045	ENDPROTT7
RT19new	3' HALFRT	1045	3' HALFRT
RT19new	ET08	647	RT19new
SK102	P6END	955	P6END
SK102	T7P6PROT	950	SK102
SP6 P66	3'HALFRT	600	ET43
SP6 P66	ET19	1312	ET43
SP6 P66	ET19	1312	ET19
V1V2-1	3' KSI-1 T7	850	V1V2-2 SP6
VPR-1	V1V2-4	1440	VPU-4
VPR-1	V1V2-4	1440	VPR-1
VPR-1	V1V2-4	1440	VPU-1
VPR-1	V1V2-4	1440	L8
VPR-1	V1V2-4	1440	V1V2-4
VPR-1	V1V2-4	1440	VPR-4
WS9REV	FGS014	1491	3' TAT-1

PCR mix.

Solution	1x (µl)
10X PCR buffer	5,0
100mM $MgCl_2$	1,0
100µM dNTPÁs	0,4
100ng/µl 5ÁPRIMER	0,5
100ng/µl 3ÁPRIMER	0,5
H_2O	37,6
5U/µl Amplitaq	0,2

- Add 5 µl of diluted product from the GAT method.

PCR Program (Perkin Elmer 9700 PCR machine).

- 5Á 95°C.
- 1Á 95°C, 1Á 55°C, 2Á 72°C for 35 cycles.
- 10Á 72°C.
- 10Á 4°C.
- 5 µl is examined on 1.0 % agarose gel and length of the PCR fragments is checked in comparison to a length marker run on the same gel.

Subsequently all PCR fragments were sequences according to the **Bigdye sequencing protocol** (Applied Biosystems) using at least the following set of sequence primers..

Primer	Sequence	Orientation	GeneID
3'P6END	TAA TAC TGT ATC ATC TGC TCC T	3'	GAG
3'T7P6 PROT	TAA TAC GAC TCA CTA TAG GGT ACT GTG ACA AGG GGT CGT TGC CA	3'	GAG
LOUW-1-GAG	TTG ACT AGC GGA GGC TAG AA	5'	GAG
V1V2-1	TGT GTA CCC ACA GAC CCC AAC CC	5'	V1V2
V1V2-2SP6	ATT TAG GTG ACA CTA TAG GAG GAT ATA ATC AGT TTA TGG GA	5'	V1V2
V1V2-4	ATT CCA TGT GTA CAT TGT ACT G	3'	V1V2
5'V3NOT	GCG CGG CCG CAC AGT ACA ATG TAC ACA TGG	5'	V3
KSIT7	TAA TAC GAC TCA CTA TAG GGT GGG TCC CCT CCT GAG GA	3'	V3
SK39	TTT GGT CCT TGT CTT ATG TCC AGA ATG C	3'	GAG
SK102	GAG ACC ATC AAT GAG GAA GCT GCA GAA TGG GAT	5'	GAG
SK431	TGC TAT GTC AGT TCC CCT TGG TTC TCT	3'	GAG
PROT-FM	CAA GGG AAG GCC AGG GAA TTT	5'	POL
SP6P66	GAT TTA GGT GAC ACT ATA GAG ATA TCA GTA CAA TGT GCT	5'	GAG
HALFRT	TAT TTC TGC TAT TAA GTC TTT TGA TGG GTC A	3'	RT
ENDPROT17	TAA TAC GAC TCA CTA TAG GGA ATA TTG CTG GTG ATC CTT TCC A	3'	POL
GAGAE-3T7	TAA TAC GAC TCA CTA TAG GGA CTA TTT TAT TTA ATC CCA GGA T	3'	GAG
VPR-1	GAT CTC TAC AIT ACT TGG CAC T	5'	VPR
VPR3-T7	TAA TAC GAC TCA CTA TAG GGA AAG CAA CAC TTT TTA CAA TAG CA	3'	VPR
VPR-4	CTT CTT CCT GCC ATA GGA GAT GCC	3'	VPR
VPU-1	GCA TCT CCT ATG GCA GGA AGA AG	5'	VPU
VPU-4	ATA TGC TTT AGC ATC TGA TGC ACA AAA TA	3'	VPU
POL5'FM	TGG AAA GGA CCA GCA AAG CTC CTC TGG AAA GGT	5'	POL
L9	CCC AAG GAA CAA AGC TCC	3'	ENV
FGS001	GTT AGT GGG AAA ATT GAA TTG GGC A	5'	RT
FGS002	AAA TTG CTT GTA ACT CAG TCT TCT	3'	RT
FGS003	ATG GGG CAG CTA ACA GGG AGA CTA	5'	RT
FGS004	TGT TTT TAC TGG CCA TCT TCC TGC T	3'	RT
FGS005	GGT AGC AGT TCA TGT AGC CAG TGG A	5'	RT
FGS006	CTT GTA TTA CTA CTG CCC CTT CAC CT	3'	RT
L8	AGA GCA GAA GAC AGT GGC	5'	VPU
L3	GGA GCA GCA GGA AGC ACT ATG	5'	ENV
L2	TAG GTA TCT TTC CAC AGC CAG	3'	ENV
FGS007	CTA ATC CTC ATC CTG TCT ACT	5'	RT
FGS008	AGT TTC GTA ACA CTA GGC AAA GGT	3'	RT
WS9REV	TAT TAA CAA GAG ATG GTG GT	5'	ENV
GPI20 3' PCR	GCT CCC AAG AAC CCA AGG AA	3'	ENV
RT 19NEW	CAC CTG TCA ACA TAA TTG GAA G	5'	RT
FGS014	CTT TTA AAA AGT GGC TAA GAT CT	3'	ENV
3'TAT-1	TTT GAA TTC TAA TCG AAT GGA TCT GTC TC	3'	ENV
ET19	GAT ATT TCT CAT GTT CAT CTT GGG CCT TAT CTA TTC C	3'	RT

Sequences that were obtained were subsequently edited and assembled by Autoassembler software. Before starting Autoassembler the sequences are edited with basic sequence analysis software in order to organise and check the raw data. The edited sequences are loaded into Autoassembler. After assemblage in Autoassembler a CONTIG is formed. This CONTIG is subsequently checked for mistakes. If a part of the sequence is not clear additional experiments have to be done. All software used is supplied by Applied Biosystems.

Example 2

In this example we isolated PBMC (peripheral blood monocytic cells) from an HIV-1 infected individual and isolated HIV-1 biological clones from these cells. PBMC were obtained from heparinized venous blood by isolation on a Percoll gradient. PBMC were suspended in Iscove's modified Dulbecco's medium supplemented with 10% DMSO, 20% fetal calf serum and antibiotics (penicillin (100 U/ml) and streptomycin (100 µg/ml)). Cells were suspended at a concentrations of approximately 5.10^6 cells/ml and aliquots of 1 ml were viably frozen and stored in liquid nitrogen until use. Cryopreserved PBMC were thawed and washed with culture medium (Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, recombinant interleukin-2 (20 U/ml, PROLEUKIN; Chiron Benelux BV) and antibiotics (penicillin (100 U/ml) and streptomycin (100 µg/ml)) to remove residual DMSO. In a 96-well plate, serial dilutions of HIV-1 infected PBMC ($0.5.10^4$ to 4.10^4 per well) were cocultivated with 2 to 3 days phytohaemagglutinin (PHA) stimulated healthy donor PBMC (10^5 per well) in a final volume of 200 µl culture medium for 28 days. For each cell dilution, multiple cocultures (28 wells) were performed. At day 7, 14, and 21, half of the culture superantants was harvested for analysis of viral p24 production using an in-house antigen capture ELISA. Cells were

resuspended and were transferred to 96-well plates containing fresh healthy donor PHA-stimulated PBMC (10^5 per well) and further cultured in a volume of 200 µl. From wells with cultures positive for p24 antigen, virus stocks were grown in 25 ml culture flasks. Cell free supernatants of these viral cultures were aliquotted and stored at -70 °C. Viruses obtained using this procedure were considered to be clonal if less than one third of the wells of a cell dilution were positive for p24.

Brief description of the drawings

Figure 1. The detected amount of HIV RNA and CD4+ T cells in a patient that carried HIV viruses with the mutations described in tables 1 through 4.

Figure 2. Growing pattern *in vitro* of viruses with the mutations described in tables 1 through 4.

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